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The Relationship of Chemical Modification of Membrane Proteins and Plasma Lipoproteins to Reduced Membrane Fluidity of Erythrocytes from Diabetic Subjects

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Summary: The significance of the two most common hallmarks of the diabetic state, hyperglycaemia and hyperlipidaemia, was investigated in terms of disorders of cell membrane dynamics. In order to examine whether the alterations in cell membrane lipid bilayer dynamics are somehow related to protein chemical modifications in plasma low- (LDL) and high-density lipoproteins (HDL) and blood cell membranes, we compared 19 poorly controlled diabetic subjects with 19 age- and sex-matched controls. The extent of (non-enzymatic) glycation, lipid peroxidation and the cholesterol/phospholipid ratio were increased in plasma low density lipoproteins and high density lipoproteins from diabetic patients. The mean steady-state fluorescence polarization values in 1,6-diphenyl-1,3,5-hexatriene-labelled isolated erythrocyte membranes from diabetic subjects were significantly greater than from control subjects (0.186 ± 0.008 vs 0.173 ± 0.006, $p < 0.001$); the fluorescence polarization values in erythrocyte membranes from diabetic and control subjects positively correlated with the extent of membrane protein glycation, lipid peroxidation and the cholesterol content. The cholesterol to phospholipid molar ratios in low density lipoproteins and high-density lipoproteins from diabetic and control subjects correlated significantly with the fluorescence polarization values in erythrocyte membranes from these subjects. Furthermore, the extent of glycation of low density lipoproteins appears to be strongly correlated with the extent of lipoprotein lipid peroxidation ($r = 0.789$, $p < 0.001$).

The atherosclerotic potential of plasma lipoproteins in diabetes mellitus was discussed in terms of membrane and plasma protein chemical modifications.

Introduction

Elevated plasma cholesterol concentrations and disturbed lipid metabolism are major risk factors in the pathogenesis of atherosclerosis and its thromboembolic complications in both the diabetic and non-diabetic populations (1). The concept that altered low density lipoproteins may have a role in the pathogenesis of atherosclerosis has emerged as a consequence of recent advances in our understanding of the mechanism involved in the interactions of lipoproteins with cells (2, 3). As a result of these interactions, a rapid change in cell membrane composition, particularly with regard to cholesterol, phospholipid, and fatty acid content, might occur, leading to alterations in membrane lipids and enzyme activities (4). Changes in erythrocyte membrane lipids and fluidity appear to be major determinants in erythrocyte elasticity and deformability (5). However, the relation between membrane-associated alterations and the formation of atherosclerotic lesions remains unclear. There is increasing evidence which indicates that non-enzymatic glycation of various proteins is an important molecular event in the diabetic state (6). There is also evidence to support the idea that many of the complications of diabetes result from hyperglycaemia (7). The observation that post-translational non-enzy-

matic glycation of proteins can occur under physiological conditions has indicated that this could be at least one mechanism through which hyperglycaemia could lead to alterations in protein structure (8, 9). Increased non-enzymatic glycation of plasma lipoproteins has been implicated in the aetiology of several complications in diabetes mellitus, including vascular disease (2, 10, 11). Other chemically or biologically modified low density lipoproteins are also poorly recognized by the low density lipoprotein receptor (11).

Recently, there has been increased interest in the idea that low density lipoproteins modified by oxidation may contribute to some of the changes associated with the formation of the atherosclerotic plaque. Several of the functional changes reported to occur when low density lipoproteins become oxidized indicate a possible role for oxidized low density lipoproteins in various pathological conditions (10, 11). The non-enzymatic glycation was correlated with the impaired dynamic properties of erythrocyte and platelet membranes from diabetic subjects (12, 13). Although their increased non-enzymatic glycation in diabetes is well recognized (7, 9, 10, 12, 13), the evidence that oxidation is also increased and accompanies the process of protein glycation has not been so far unambiguously proven in diabetic subjects.

In the present study, we determined the influence of diabetes on erythrocyte membrane fluidity and on the cholesterol to phospholipid molar ratio in erythrocyte membranes and in plasma lipoproteins. We also compared the extent of non-enzymatic glycation and accumulation of lipid peroxidation products in erythrocyte membranes and apoproteins of low density lipoproteins and high density lipoproteins in diabetic and control subjects, in order to determine the influence of possible chemical modifications of proteins on red blood cell membrane dynamics.

Materials and Methods

Subjects

Nineteen non-fasting diabetic patients (13 men: 11 type I insulin-dependent, 2 type II non-insulin-dependent; 6 women: 5 type I, 1 type II with a mean ± SD age of 36 ± 10 years (range 23–53) and 19 age- (36 ± 10 years, range 22–57) and sex-matched control subjects were studied. Mean body weights were not significantly different between control (70.7 ± 11.9 kg) and diabetic (71.4 ± 10.0 kg) subjects. Mean duration of diabetes was 18 ± 9 years. Of the diabetic subjects, 17 had been treated with insulin alone and 2 had been treated with diet and an oral hypoglycaemic agent (Glyburide). None of the diabetic or control subjects was treated with any normolipidaemic medication. Two diabetic subjects had retinopathy, 1 had angina pectoris and 3 had hypertension. Blood was collected from each diabetic subject and the paired control subject on the same day. These studies were approved by the Committee on the Ethics of Research in Human Experimentation at McMaster University.

Blood collection and preparation of erythrocyte membranes

Blood (100 ml) was collected into a plastic syringe from a forearm vein through an 18 gauge needle. Fourteen millilitres of this blood were added to EDTA-containing vacutainer tubes (Becton Dickinson, Missisauga, Ontario, Canada) for determination of HbA_{1c} and glucose concentration of the plasma. The remaining blood was anticoagulated with acid-citrate-dextrose solution (1 ACD + 6 blood, vol/vol) and used for preparation of erythrocyte membranes. Red blood cells washed four times with phosphate-buffered saline pH 7.4 were subjected to moderate haemolysis in Tris-HCl/$EDTANa_2$ buffer pH 7.0 according to *Marchesi & Palade* (14). The isolated erythrocyte membranes were resuspended in ice-cold phosphate-buffered saline with phenylmethylsulphonyl fluoride, sodium azide and $EDTANa_2$. The protein content in erythrocyte membrane suspensions was measured according to the modified method of *Lowry* et al. (15). The membrane suspensions were subsequently used for determination of membrane fluidity, cholesterol, phospholipid, lipid peroxidation and non-enzymatic glycation of membrane protein. The samples from diabetic and control subjects were obtained, stored and analysed at the same time and under the same conditions.

Isolation of low-density lipoproteins and high-density lipoproteins from plasma

In order to inhibit protease and lipase activities, plasma was supplemented with 100 ml/l of a solution containing phenylmethylsulphonyl fluoride (2 mmol/l), $EDTANa_2$ (1 mmol/l), sodium azide (2 mmol/l). High density lipoproteins were separated from low density lipoproteins by the precipitation of the latter using a combination of sodium phosphotungstate and magnesium chloride (16), after prior separation of very low density lipoproteins according to *Schriewer* et al. (17). Low density lipoproteins and high density lipoproteins were purified according to *Mills* et al. (16) and extensively dialysed against phosphate-buffered saline with phenylmethylsulphonyl fluoride/$EDTANa_2$/NaN_3 pH 7.2 at 4 °C. Ten microlitres of β-hydroxytoluene in methanol (0.1 mol/l) were added per 1 ml of lipoprotein solutions to protect samples from spontaneous peroxidation, and the samples were stored at −70 °C until assayed.

Fluidity measurements

Erythrocyte membrane fluidity was determined by measuring fluorescence polarization (18) with the hydrophobic probe 1,6-diphenyl-1,3,5-hexatriene (Molecular Probes, Eugene, OR, USA; 0.5 mmol/l in tetrahydrofuran). 1,6-Diphenyl-1,3,5-hexatriene was diluted 1 : 100 in phosphate buffered saline, pH 7.4, and vigorously mixed immediately before use. One volume of this diluted 1,6-diphenyl-1,3,5-hexatriene dispersion was added to 1 vol of the erythrocyte membrane suspension containing 0.2 g/l protein, and the mixture was incubated at 37 °C for 35 min. The final protein concentration was 0.1 g/l.

Steady-state fluorescence polarization was measured at 37 °C with a Perkin-Elmer spectrofluorometer equippped with polarizers in the excitation and emission beams. The excitation and emission wavelengths were 360 and 430 nm, respectively. Fluorescence polarization was determined using a standard formula from emission intensities that were polarized parallel and perpendicular to the direction of the polarized excitation (18). The grating transmission factor of *Chen & Bowman* (19) was used to correct for the depolarization effect of grating monochromators (20). A fluorescence intensity value for a non-labelled blank was subtracted as a correction for scattered light. Fluorescence polarization values mainly reflect lipid structural order, and lipid fluidity has been defined as the reciprocal of the lipid structural order parameter (21).

Measurement of cholesterol and phospholipids

Samples of the erythrocyte membrane suspensions containing 100–200 μg protein were subjected to lipid extraction using a two-step extraction procedure with neutral solvent (chloroform : methanol, 1 + 2, vol/vol) and acidic solvent (chloroform : methanol : HCl : water, 3 +4 + 0.5 + 0.1, vol/vol) (22). The lipid extracts were dried under nitrogen and lipid residues were re-dissolved in chloroform (1 ml). The cholesterol content of lipid extracts of erythrocyte membranes was determined by the cholesterol oxidase fluorometric assay (23), and phosphorus was measured using ammonium molybdate and ascorbic acid after mineralization with magnesium nitrate (24).

Measurement of glycation

The extent of glycation was determined as described earlier (25). Aliquots of erythrocyte membranes or isolated lipoproteins containing 100 μg or 2 mg protein, respectively, were treated with equal volumes of 24 mmol/l sodium borohydride-[^{3}H] (New England Nuclear, Mississauga, Ontario, Canada; 3.7 TBq/mol = 100 mCi/mmol) and incubated at 37 °C for 2 h. After incubation, 30 g/l bovine serum albumin was added to each sample and the protein precipitated with 100 g/l trichloroacetic acid. The pellet was dissolved in 1 mol/l NaOH and reprecipitated with trichloroacetic acid. This procedure was repeated 4–5 times until no further radioactivity could be detected in the supernatant. Radioactivity was measured in the final pellet redissolved in 1 mol/l NaOH. Results were expressed in nmol glucose assuming that 1 mol of glucose moiety adduct is reduced by 1 mol of sodium borohydride-[^{3}H].

Determination of HbA_{1c} and plasma glucose concentrations

HbA_{1c} was determined in whole blood using a glycoaffinity column (26) (the normal range of HbA_{1c} is 4.5%–7.5%). Plasma glucose was measured by the glucose oxidase method with an Ektachem (Eastman-Kodak, Rochester, NY, USA) (27).

Measurement of lipoperoxidation products

The products of lipid peroxidation in erythrocyte membranes and in plasma lipoproteins were determined according to the method of *Wong* et al. (28) with modifications. In principle, the adduct of malondialdehyde with 2-thiobarbituric acid was separated from interfering chromogens on the HPLC column and lipoperoxide concentrations were computed by reference to a calibration curve prepared by assaying tetraethoxypropane. Before addition of thiobarbituric acid reagent the incubation mixture was supplemented with β-hydroxytoluene and the solubilizing agent, polyoxyethylene ether W-1 (29) (Sigma Chemical Co.), at the final concentrations of 3 mmol/l and 1 g/l, respectively. The amounts of lipoperoxides were expressed in nmol of thiobarbituric acid reacting substance.

Analysis of data

Means ± SD are given. Paired t tests (2-tailed) were used to determine the significance of differences. The normal distribution of data was confirmed using the *Shapiro-Wilk*'s test (30). A combined test for correlation coefficients was performed to assess the relationships among the quantities investigated (31).

Results

The mean plasma glucose and HbA_{1c} were significantly greater in diabetic patients than in control subjects (2.12 ± 0.96 g/l vs 0.84 ± 0.16 g/l, $p < 0.001$, and 12.5% ± 3.0% vs 5.8% ± 0.5%, $p < 0.001$, respectively).

The mean steady-state fluorescence polarization value of 1,6-diphenyl-1,3,5-hexatriene in isolated erythrocyte membranes from diabetic subjects (0.186 ± 0.008, n = 14) was significantly greater than that for control subjects (0.173 ± 0.006, n = 14, $p < 0.001$). The lipid contents of erythrocyte membranes and of low-density lipoproteins and high-density lipoproteins are shown in table 1. The amount of cholesterol and the cholesterol to phospholipid molar ratio were significantly higher in isolated erythrocyte membranes from diabetic subjects compared with control subjects, but the phospholipid contents were similar. In plasma low-density lipoproteins from di-

Tab. 1. Cholesterol and phospholipid contents of erythrocyte membranes and of plasma low-density lipoproteins and high-density lipoproteins from diabetic and control subjects.

	Diabetic	Control	Significance of difference p <
Erythrocyte membranes			
Cholesterol (μmol/mg protein)	0.470 ± 0.039	0.453 ± 0.049	0.04
Phospholipid (μmol/mg protein)	0.827 ± 0.063	0.830 ± 0.085	ns
Cholesterol/phospholipid molar ratio	0.571 ± 0.077	0.551 ± 0.080	0.03
LDL			
Cholesterol (μmol/mg protein)	5.33 ± 0.72	4.73 ± 0.47	0.025
Phospholipid (μmol/mg protein)	1.17 ± 0.20	1.34 ± 0.14	0.025
Cholesterol/phospholipid molar ratio	4.67 ± 0.87	3.56 ± 0.57	0.001
HDL			
Cholesterol (μmol/mg protein)	1.09 ± 0.15	1.12 ± 0.15	ns
Phospholipid (μmol/mg protein)	0.56 ± 0.09	0.65 ± 0.05	0.035
Cholesterin/phospholipid molar ratio	2.02 ± 0.41	1.74 ± 0.22	0.02

Values are means ± SD for 14 subjects in each group.

abetic subjects, the concentration of cholesterol was signficiantly higher ($p < 0.025$), and that of phospholipid significantly lower ($p < 0.025$) than the corresponding values for control subjects. In the plasma high density lipoproteins, the cholesterol content was not significantly different between groups, whereas the amount of phospholipids was significantly lower ($p < 0.035$) in diabetic subjects compared with control subjects. As a result of these differences in low density lipoproteins and high density lipoproteins, the cholesterol to phospholipid molar ratios were significantly higher in low density lipoproteins ($p < 0.001$) and high density lipoproteins ($p < 0.02$) from diabetic subjects compared with control subjects.

The amounts of non-enzymatically attached glucose were significantly increased, both in erythrocyte membranes ($p < 0.001$) and in plasma low-density lipoproteins ($p < 0.001$) and high-density lipoproteins ($p < 0.015$) from diabetic subjects compared with control subjects (tab. 2); the most significant differences occurred in erythrocyte membranes.

The levels of thiobarbituric acid reacting substances, which are the hallmark of lipid peroxidation, were

Tab. 2. The extent of non-enzymatic glycation of proteins in erythrocyte membranes and in plasma low density lipoproteins and high density lipoproteins from diabetic and control subjects.

	Diabetic	Control	Significance of difference p <
Erythrocyte membranes (nmol glucose/mg protein)	25.63 ± 3.49	21.23 ± 2.78	0.001
LDL (nmol glucose/mg protein)	29.05 ± 4.53	22.19 ± 2.83	0.001
HDL (nmol glucose/mg protein)	34.23 ± 3.71	29.72 ± 4.67	0.015

Values are means ± SD for 13–14 subjects in each group.

Tab. 3. Lipoperoxide adducts in erythrocyte membranes and plasma low density lipoproteins and high density lipoproteins from diabetic and control subjects in nmol of thiobarbituric acid reactive substance per mg of protein.

	Diabetic	Control	Significance of difference p <
Erythrocyte membranes	4.17 ± 0.91	2.97 ± 0.81	0.025
LDL	0.241 ± 0.035	0.207 ± 0.020	0.01
HDL	0.217 ± 0.056	0.204 ± 0.047	0.03

Values are means ± SD for 11 subjects in each group.

Tab. 4. Correlation coefficients among the quantities examined in diabetic and control subjects.

Quantity	Correlation coefficient (r)	Significance of correlation
Erythrocyte membrane fluorescence polarization:		
vs non-enzymatic glycation of erythrocyte membrane proteins	0.399	$p < 0.04$
vs lipid peroxidation in erythrocyte membranes	0.551	$p < 0.008$
vs cholesterol content in erythrocyte membranes	0.363	$p < 0.05$
vs cholesterol/phospholipid (molar ratio) in LDL	0.590	$p < 0.003$
vs cholesterol/phospholipid (molar ratio) in HDL	0.371	$p < 0.05$
Non-enzymatic glycation of LDL:		
vs lipid peroxidation in LDL	0.789	$p < 0.003$
vs phospholipid content in LDL	−0.408	$p < 0.03$
vs cholesterol/phospholipid (molar ratio) in LDL	0.476	$p < 0.015$
Erythrocyte cholesterol content:		
vs cholesterol content in LDL	0.381	$p < 0.05$
vs non-enzymatic glycation of HDL	0.372	$p < 0.05$
Lipid peroxidation in LDL:		
vs lipid peroxidation in erythrocyte membranes	0.613	$p < 0.03$
vs cholesterol content in LDL	0.479	$p < 0.035$

Correlation coefficients are determined from 11–14 diabetic and 11–14 control subjects.

increased in erythrocyte membranes and plasma lipoproteins in diabetics subjects; the most highly significant increase was observed in low-density lipoproteins ($p < 0.01$) (tab. 3).

The degrees of correlation between the various quantities examined were determined for diabetic and control subjects (tab. 4). Fluorescence polarization values in erythrocyte membranes significantly correlated with the extent of non-enzymatic glycation, lipid peroxidation and the cholesterol content in these membranes and also with the cholesterol to phospholipid molar ratios in plasma low density lipoproteins and high-density lipoproteins. In low density lipoproteins the extent of non-enzymatic glycation significantly correlated with peroxidation and the cholesterol to phospholipid molar ratio and inversely correlated with the phospholipid content. The cholesterol content of erythrocyte membranes significantly correlated with the cholesterol content in low density lipoproteins and the extent of non-enzymatic glycation in high density lipoproteins. Lipid peroxidation in low density lipoproteins significantly correlated with lipid peroxidation in erythrocyte membranes and with the cholesterol content in low density lipoproteins. Other correlations were not statistically significant.

Discussion

The results from the present study show that, compared with erythrocyte membranes from controls, erythrocyte membranes from diabetic subjects display reduced membrane lipid fluidity, together with the increase in membrane protein glycation, lipid peroxidation, and the cholesterol to phospholipid molar ratio. Furthermore, low density lipoproteins and high density lipoproteins from diabetic subjects were more extensively glycated, had a higher cholesterol to phospholipid molar ratio, and contained greater amounts of lipid peroxidation products than those from control subjects. The extent of non-enzymatic glycation correlated with the extent of lipid peroxidation and the cholesterol to phospholipid molar ratio in low density lipoproteins from diabetic and control subjects, but this correlation was not evident in high density lipoproteins or erythrocyte membranes. The values for membrane lipid fluidity in erythrocytes from diabetic and control subjects inversely correlated with the cholesterol to phospholipid molar ratios in low density lipoproteins and high density lipoproteins.

In our recent study, conducted within the same population of patients, we also found a significant inverse correlation between membrane lipid fluidity and the extent of non-enzymatic glycation of membrane proteins in platelets from diabetic and control subjects, although the cholesterol to phospholipid molar ratio did not differ between platelets from diabetic and control subjects (13). It seems reasonably certain, therefore, to conclude that red blood cells are not unique with respect to the rigidization of the membrane lipid bilayer due to the increased membrane protein glycation in the diabetic state. In platelets, non-enzymatic glycation of membrane proteins also results in the rigidification of the lipid bilayer.

Non-enzymatic glycation of low density lipoproteins has been reported to alter the binding of low density lipoproteins to platelets and to various other cell types (32, 33). In some diabetics the level of glycation may be sufficient to interfere with the normal metabolism of low density lipoproteins (25). The extent of the glycation of low density lipoproteins in diabetic juveniles has been previously reported to correlate with lipid composition of low density lipoproteins and the cholesterol to phospholipid molar ratio in erythrocyte membranes. These changes seemed to invoke the alterations in membrane lipid bilayer dynamics in erythrocytes from diabetic subjects (12). Thus, non-enzymatic glycation of erythrocyte membrane proteins seems to be of major importance in the impairment of red blood cell functions; additionally it appears to be involved in the augmentation of the rate of post-synthetic modifications of plasma low density lipoproteins. Increased glycation of low density lipoproteins, either in vitro or in material isolated from diabetic subjects, may lead to impaired uptake by the classic low density lipoprotein receptor and the increased uptake by human monocyte-derived macrophages (for reviews see (11, 34)). The former effect may increase circulating low-density lipoprotein concentrations, whereas the latter effect may contribute to foam cell formation, a precursor in the development of atherosclerosis (9–11). Furthermore, a positive correlation between the degree of non-enzymatic glycation of low density lipoproteins and the enhancement of platelet aggregation has been demonstrated, and numerous data suggest that glycated low density lipoproteins may contribute to the hyperaggregability of platelets in diabetics (32).

In the present study, we found significantly increased cholesterol to phospholipid molar ratios in plasma low density lipoproteins and high density lipoproteins and in erythrocyte membranes from diabetic subjects, and these changes were inversely related to the membrane lipid fluidity of erythrocytes. Our present study demonstrates that erythrocyte membranes, as well as plasma low density lipoproteins and high density lipoproteins from diabetic subjects, contain increased

levels of thiobarbituric acid reacting substances compared with control subjects. These changes were associated with a reduced membrane fluidity of erythrocytes from diabetic subjects compared with control subjects, which has been taken as evidence that lipid peroxidation may be essentially involved in the modification process (35, 36). The concept is supported by the significant inverse correlation found among erythrocyte membrane fluidity and the extent of lipid peroxidation in erythrocyte membranes and plasma low density lipoproteins and high density lipoproteins. This relation does not appear to have been examined previously, but the reduced erythrocyte membrane fluidity in the diabetic state is consistent with our earlier reports, in which we showed a reduced membrane fluidity in isolated erythrocyte membranes from diabetic adults and juveniles, and the alterations were found to relate both to the membrane lipid composition (37, 38) and to the extent of membrane protein glycation (12, 39).

It has been shown that native and oxidatively modified lipoproteins differ importantly in their effects on cells. The oxidized low density lipoproteins and high density lipoproteins are more reactive than the native ones, and they are thought to promote the vascular events which are characteristic of atherogenesis (2, 11). Our results show that the increased lipid peroxidation of erythrocyte membranes, low density lipoproteins and high density lipoproteins occurred in all but 1, 2, and 3 diabetic subjects, respectively. With respect to this finding our results do not agree completely with the results of the studies published hitherto. There is no direct evidence that lipid peroxidation is increased in uncomplicated human diabetes (40–42). With the exception of one report by *Collier* et al. (43), the evidence suggests that peroxidation of circulating plasma lipoproteins may not be related to diabetes itself, but rather to the underlying diabetic complications.

The positive correlation between the extent of glycation and thiobarbituric acid reacting substances level is not obscure, since the process of non-enzymatic glycation has been postulated to augment free radical formation. This phenomenon may be of particular importance in diabetic patients, where the sources of free oxygen radicals are augmented by the increased glycation of proteins, so that natural defences may be overwhelmed (35, 36).

The formation of reactive aldehydes is known to impair the physical properties and the flexibility of red blood cell membranes (44). This seems to be the case in the present study, as we found a very significant inverse correlation between erythrocyte membrane fluidity and the extent of erythrocyte membrane lipid peroxidation.

The above reported observations suggest the potential importance of glycated and oxidized low density lipoproteins in the pathogenesis of atherosclerosis and adds a new dimension to the relationship between plasma lipoproteins and blood cell membranes in diabetes, as well as implying a potential role of altered lipoproteins in the pathogenesis of atherosclerosis.

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